

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : C12P 19/04, C12N 1/20, A23L 1/24 // (C12N 1/20, C12R 1:225)		A1	(11) International Publication Number: WO 94/12656 (43) International Publication Date: 9 June 1994 (09.06.94)
(21) International Application Number: PCT/EP93/03338		(81) Designated States: AT, AU, BB, BG, BR, BY, CA, CH, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, KZ, LK, LU, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SK, UA, US, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).	
(22) International Filing Date: 26 November 1993 (26.11.93)			
(30) Priority Data: 92203728.8 2 December 1992 (02.12.92) EP		(34) Countries for which the regional or international application was filed: NL et al.	
(71) Applicant (for all designated States except US): QUEST INTERNATIONAL B.V. [NL/NL]; Huizerstraatweg 28, NL-1411 GP Naarden (NL).		Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(72) Inventors; and			
(75) Inventors/Applicants (for US only): VAN DEN BERG, Dirk, Johannes, Cornelis [NL/NL]; Montgomerylaan 60, NL-2622 PP Delft (NL). LEDEBOER, Adrianus, Marinus [NL/NL]; B. Lefevre de Montignyplein 8, NL-3055 ML Rotterdam (NL). ROBIJN, Gerard, Willem [NL/NL]; Dirkje Mariastraat 16, NL-3551 SL Utrecht (NL). VREEKER, Robert [NL/NL]; Ratelaar 20, NL-3248 BN Melissant (NL).			
(74) Agent: JOPPE, Hermina, L., P.; Unilever N.V., Patent Division, P.O. Box 137, NL-3130 AC Vlaardingen (NL).			

(54) Title: **LACTOBACILLUS SAKE LIKE STRAINS, PRODUCTION AND USE OF THEIR EXOPOLYSACCHARIDES**

(57) Abstract

New *Lactobacillus sake* like strains are provided obtainable from meat products, which strains are capable of producing an exopolysaccharide having shear-thinning properties, even at low concentrations, and/or thickening and/or emulsion-stabilizing properties.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BH	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

LACTOBACILLUS SAKE LIKE STRAINS, PRODUCTION AND USE OF
THEIR EXOPOLYSACCHARIDES

Background of the invention

5

A large number of food products like mayonnaises, dressings, margarines, spreads or low-fat or zero-fat substitutes, can be stabilized by polysaccharides as emulsion stabilizers or thickening agents. Also in the 10 medical, pharmaceutical and cosmetic fields, polysaccharides are used as emulsion stabilizers. Well known polysaccharides are obtained from a variety of plant seeds, e.g. guar gum from *Cyamopsis tetragonoloba* (guar), or locust bean gum (LBG) from locust bean. Other well known 15 sources are seaweeds, giving carrageenan, alginates or agar.

One disadvantage of all these polysaccharides is, that the supply of the sources and thus the constant availability of 20 the polysaccharides, is not certain, while demands are ever growing. This has already led to high and fluctuating prices for a highly functional polysaccharide like LBG. As an option to produce a product at reasonable prices the process described in

25

EP-B-0121960 (UNILEVER) was developed for converting the cheaper, but less versatile polysaccharide guar gum into a clearly improved gum. A potentially commercial production of the required guar α -galactosidase was described in 30 WO-A-87/07641 (UNILEVER) = US-5082778.

Another disadvantage is that the isolation procedure for polysaccharides from seeds or seaweed is rather cumbersome.

35 A further disadvantage is that most of the polysaccharides have not the highly wanted, non-Newtonian property of shear-thinning, which is the effect that the viscosity reversibly decreases as the shear is enhanced, even at low

concentrations.

A polysaccharide that both has the wanted shear-thinning property and is reliable with respect to production and 5 isolation, is xanthan gum, a polysaccharide produced by the bacterium *Xanthomonas campestris* through fermentation. Consequently, xanthan gum is increasingly used in food and medical products and is expected to have by far the highest growth potential in the coming years. However, the 10 producing microorganism, *Xanthomonas campestris*, is not food-grade. Moreover, a food product containing added xanthan gum has often to be labelled as containing an additive. This is disadvantageous in view of the present trends towards healthy "green" or only scarcely labelled 15 food products.

Thus a need exists for a polysaccharide produced by a food-grade microorganism, having properties similar to or even superior to xanthan gum. Such a polysaccharide can either 20 be added to the food product and the resulting product has to be labelled (but then the product is a so-called "friendly labelled" additive), or it can be produced *in situ* without the necessity of any labelling, because the microorganism is food-grade.

25

Summary of the invention

The present invention is based on the results of a large screening program involving about 600 lactic acid bacteria 30 strains present in various food products, e.g. olives, traditional cheeses, and sour dough, which screening program resulted in the isolation of 30 exopolysaccharides-producing strains having thickening and/or emulsion-stabilizing properties. However, only some of these 30 35 lactobacilli, which appeared to be present in meat products, especially in Belgian sausages, produce exopolysaccharides (EPS) having (1) thickening and/or (2) emulsion stabilizing properties and/or even show (3) the

highly desirable shear-thinning property, in particular at low concentrations. As an example thereof a *Lactobacillus* strain was isolated, characterised as a *Lactobacillus sake* like strain and given the name *Lactobacillus sake* like strain 0-1. This strain, combining the three different properties mentioned above, was deposited under the conditions of the Budapest Treaty at the Centraalbureau voor Schimmelcultures in Baarn, The Netherlands with number CBS 532.92.

10

The present invention relates to an EPS obtainable from said *Lactobacillus sake* like strain 0-1 as well as to exo-polysaccharides obtainable from similar *Lactobacillus sake* like strains having either viscosity-increasing properties, 15 or shear-thinning properties, or both. The EPS obtainable from *Lactobacillus sake* like strain 0-1 comprises units of the monosaccharides rhamnose and glucose.

20 The invention also relates to a *Lactobacillus sake* like strain capable of producing such EPS. A preferred strain is the *Lactobacillus sake* like strain 0-1, deposited as CBS 532.92.

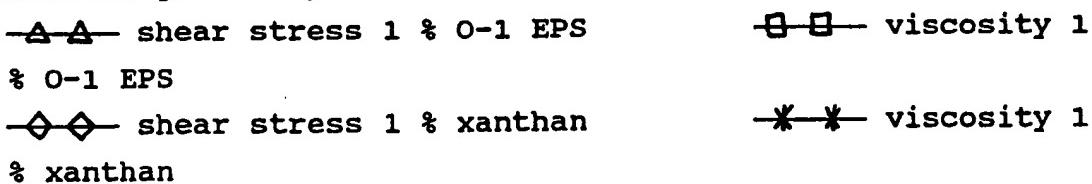
25 The invention further relates to a process for producing such EPS comprising (a) growing a *Lactobacillus sake* like strain capable of forming an EPS in a suitable medium under conditions whereby said EPS is formed, and optionally (b) isolating the EPS formed. In one embodiment of this aspect 30 of the invention such process can be used for the production of an EPS or an EPS-containing product, either of which can be used as an additive for food products or in medical, pharmaceutical and cosmetic applications. In another embodiment the process can be used for *in situ* production of an EPS comprising growing a *Lactobacillus sake* like strain in a dairy liquid medium under conditions 35 whereby said EPS is formed until the culture has a relative high density of said lactobacilli. Preferably the product obtained by said process is not thereafter subjected to an

intensive shear treatment. Such culture containing an EPS can advantageously be incorporated into dairy ingredient-containing products such as dressings, margarine, mayonnaise, and spreads, and low-fat or zero-fat
5 substitutes therefor.

Brief description of the Drawings

In Figure 1 the UPGMA (Unweighted Pair Group Method using
10 Average linkage) dendrogram of the Pearson Product Moment Correlation coefficients ($r \times 100$) between SDS-PAGE protein patterns is given. The strains assigned with a "LAB"-number are stored in the closed "Lactic Acid Bacteria" culture collection of the University of Ghent. Strains assigned
15 with a "LMG"-number belong to the "Laboratorium voor Microbiologie, Gent" culture collections. The reference strains used in this study are indicated in the dendrogram, as are the EPS-producing strains. The strain "LAB28" (O-1) refers to the *Lactobacillus sake* like strain O-1 with
20 deposit number CBS 532.92.

In Figure 2 the shear rate versus viscosity and shear rate versus shear stress of a 1 % (wt./vol.) aqueous solution of both the EPS from *Lactobacillus sake* like strain O-1 and
25 xanthan gum are plotted.


—▲— shear stress 1 % O-1 EPS —□— viscosity 1
 % O-1 EPS
—◇— shear stress 1 % xanthan —*— viscosity 1
 % xanthan

30 In Figure 3 the structure of a preferred EPS according to the invention is provided.

Detailed description of the invention

35

In one aspect the invention relates to a polysaccharide that can be produced by a *Lactobacillus sake* like strain, isolated from a meat product, e.g. a Belgian sausage. Thus

the invention provides a polysaccharide obtainable from a food-grade *Lactobacillus sake* like strain. One preference is for a polysaccharide comprising units of the monosaccharides rhamnose and glucose, more preferably in a 5 ratio of about 1:4 to about 1:1 most preferably in a ratio of 3:7 to 1:1.

It has been found that the EPS obtainable from *Lactobacillus sake* generally comprises acetyl and phospho 10 glycerol side groups. The EPS is believed to be partially O-acetylated, whereby the level of acylation is from 0.40 to 0.45 per rhamnose unit for example 0.425 per rhamnose unit. The level of phosphorylation is believed to be more than 0.45 per rhamnose unit, for example 0.50 per rhamnose unit. 15 Figure 3 gives the most likely structure as obtained by NMR techniques of the EPS obtained from *Lactobacillus sake* O-1. The EPS is composed of repeating pentasaccharide units with 3 glucose units and 2 rhamnose units, whereby one of the rhamnose units has a 1-phospho glycerol substituent and the 20 other rhamnose unit contains on average about 0.85 O-acetyl groups.

For some embodiments of the invention it may be useful to reduce the number of substituent groups. This may be 25 accomplished by conventional chemical techniques, for example the EPS may be O-deacylated by mild alkalic treatment, for example with ammonia. also the EPS may be subjected to a highly alkaline treatment (NaOH) for further O-deacetylation and dephosphorylation. It is believed that 30 removal of the side-groups may influence the rheology properties of the EPS.

Another preference is that the polysaccharide according to the invention gives a viscosity of at least 10 mPa.s when 35 measured with a Haake Rotovisco RV100 viscosimeter at a concentration of 2 g/l (= 0.2 % wt.) in water and a shear rate of 300 s⁻¹, or a viscosity of at least 1000 Mpa.s when measured at a concentration of 10 g/l (= 1 % wt.) in water

and a shear rate of 3 s^{-1} . Especially preferred is a viscosity of more than 100 Pa.s at a shear rate of 0.01 s^{-1} and a concentration of 1 wt% in water.

- 5 Other preferred rheology characteristics of the EPS of the invention are: a value p of between 0.7 and 0.9, wherein the viscosity (Pa.s) \approx (shear rate (s^{-1}))^{-p}. Other characteristics are: viscosity of 1wt% solution at 0.01 s^{-1} is relatively T-independent (less than 50% fluctuation in 10 the range 4 to 20°C), pH independent (less than 10% fluctuation in pH range 4-6) and relatively independent on the presence of salts. Furthermore it has been observed that a synergistic increase in viscosity can be observed if the EPS of the invention is combined with guar gum or LBG.
- 15 Also the EPS of the invention tend to act as a thickener rather than a gelling agent. This difference can be shown by shear modulus measurements.

Another preferred feature of a polysaccharide according to 20 the invention is, that it has shear-thinning properties in that the viscosity is reversibly reduced upon increasing the shear rate. A most preferred polysaccharide is one that is obtainable from *Lactobacillus sake* like strain O-1 deposited as CBS 532.92. This latter polysaccharide has the 25 following properties:

External appearance: tasteless and odourless white powder;

Solubility: readily soluble in water, scarcely soluble in methanol, ethanol and acetone;

Composition: 60-65 % glucose units and 35-40 % rhamnose 30 units.

Viscosity: measured in an aqueous solution at 25 °C for both EPS from *Lactobacillus sake* like strain O-1 and xanthan as comparison

viscosity in mPa.s

35	<u>concentration</u>	<u>shear rate</u>	<u>O-1 EPS</u>	<u>xanthan</u>
	0.2 % wt.	300 s^{-1}	18	14
	1.0 % wt.	3 s^{-1}	2440	1240

In another aspect the invention provides a process for producing a polysaccharide according to the invention, which process comprises (a) growing a *Lactobacillus sake* like strain capable of forming an EPS in a suitable medium under conditions whereby said EPS is formed, and optionally (b) isolating the EPS formed. A suitable medium appeared to be a so-called Semi Defined Medium (SDM) consisting of a carbon source like glucose or another fermentable carbohydrate, at least one nitrogen source, a phosphate source, e.g. a mixture of Na_2HPO_4 and KH_2PO_4 in such a concentration that it also has a high buffering capacity, vitamins, minerals (especially Mn^{2+}), amino acids and peptide mixtures. Two SDM's are given in Examples 1 and 6. As will be apparent to a skilled person these SDM's for laboratory trials need to be adapted when commercially producing the EPS at a larger scale. The strain is generally grown between 15 °C and 40 °C for 24 to 48 hours, without aeration for optimal production of the EPS. The EPS can be isolated from the fermentation broth by any suitable technique e.g. by means of a precipitation process using an organic solvent in which the EPS is not soluble or has a limited solubility. Another possibility is removal of the water, e.g. by evaporation, as is often done in the production of xanthan gum on a commercial scale, or by membrane filtration.

The thus isolated polysaccharide can be applied as such as an additive to food products. For convenience and easy handling, it is however sometimes preferred to apply the polysaccharide to a carrier material. This can be accomplished by any suitable technique. Any edible carrier material can be used, although the use of protein carriers e.g. whey protein or soy protein is especially preferred. The polysaccharide can be applied as a "friendly" labelled additive in the production of dressings, mayonnaise, spreads and their low-fat and zero-fat equivalents, creams, sauces, meat (products) cheese, puddings, ice-cream and

bakery products, using processes familiar to those skilled in the art, which will be further illustrated in the Examples. The level of polysaccharide will generally be from 0.01 to 15 wt%, more preferred 0.1 to 10 wt%, most 5 preferred 0.5 to 5 wt%.

For some of these applications (e.g. cheese) it is preferred that the product is prepared under relatively quiescent conditions, e.g. gentle or no stirring after 10 mixing the ingredients in order to avoid undesired shear-thinning.

Thus the invention also provides a process for producing a food product containing a thickened aqueous phase, which 15 process comprises incorporating into said food product an effective amount of a polysaccharide according to the invention.

Another embodiment of this aspect of the invention which 20 does not require labelling the food product as containing an additive, is a process in which the EPS is formed by the *Lactobacillus sake* like strain during fermentation of a commercially acceptable medium for starter cultures, after which the water can be removed by conventional techniques, 25 e.g. evaporation, membrane filtration, or spray-drying.

Labelling is also not required when the polysaccharide is produced *in situ* by a food-grade micro-organism. An example is a process for producing a mayonnaise type product, in 30 which milk or a milk-based medium, e.g. pasteurised skimmilk containing Hysoy (ex Quest Bioproducts, U.S.A.) and MnSO₄, is fermented with the *Lactobacillus sake* like strain at 30 °C for 18 hours until a sufficient high amount 35 of lactobacilli is formed producing sufficient EPS. Subsequently the fermented milk product is carefully mixed both with an aqueous phase containing usual ingredients, e.g. salt, sugar, acids, flavour components and starch, and with a separately prepared mayonnaise pre-emulsion phase.

Thus the invention also provides a process for *in situ* producing a polysaccharide according to the invention, comprising growing a *Lactobacillus sake* like strain in an edible medium e.g. a dairy liquid medium under conditions 5 whereby said EPS is formed until the number of said lacto-bacilli is in the order of 10^7 to 10^{11} per ml, preferably 10^9 to 10^{10} per ml. Preferably the product obtained by said process is not subsequently subjected to an intensive shear treatment.

10

Another embodiment of this aspect of the invention is a process for producing a product containing a dairy ingredient such as dressings, margarine, mayonnaise, and spreads, and low-fat or zero-fat substitutes therefor, 15 which comprises incorporating into said product a liquid dairy medium obtained by a process for *in situ* producing a polysaccharide according to the invention. The dairy ingredient can form the total aqueous phase or at least part of the aqueous phase of said product.

20

The invention is further illustrated by the following Examples, which do not limit the scope of the present invention. Percentage in the following Examples are expressed as wt. unless otherwise stated.

25

30

Example 1 Search for exopolysaccharide-producing lactic acid bacteria present in sausages

From 5 different home-made, traditionally fermented, 5 chouriço sausages from the region of Alenteijo in Portugal and from a traditionally fermented salami sausage from Recogne in Belgium, 10 g of sausage was suspended in 90 ml of 0.85 % NaCl solution using a stomacher model BA6021 (ex Seward Laboratory). These suspensions were diluted 10⁻², 10 10⁻⁴ and 10⁻⁶ times in 0.85 % NaCl and 0.1 ml of the respective dilutions was plated out on MRS-agar which contained 20 mg pimafucine (ex Duchefa) per 100 ml medium to prevent growth of yeasts or fungi. The plates were incubated anaerobically at 30 °C for 48 hours.

15 In total 159 lactic acid bacteria strains were isolated. All strains were selected for the production of EPS as follows. The individual strain was grown overnight in MRS-broth at 30 °C. "EPS selection medium" (ESM), composed of 9 20 % skimmilk powder (ex Frico Domo), 0.35 % Yeast Extract (ex Difco), 0.35 % Bacto Pepton (ex Difco) and 1 % glucose was inoculated with 1 % (v/v) of this overnight culture. After 24 hours incubation at 30 °C, the milk-curd was checked for "ropiness". To this end 1 ml of the milk-curd was taken up 25 in a 1 ml volumetric pipet. The pipet was emptied again and when drops falling from the tip of the pipet showed a slimy behaviour, this referred to EPS-production in the ESM. With this method 4 of the 159 lactic acid bacteria strains isolated turned out to be EPS producers. These 4 strains 30 were all isolated from the Belgium salami, whereas no EPS-producing strains could be isolated from the Portuguese sausages investigated.

The viscosity produced by a 1 % (v/v) inoculum of an 35 overnight grown culture (in MRS broth) in a Semi Defined Medium (SDM-1) (containing 2.5 g K₂HPO₄, 3.0 g KH₂PO₄, 0.6 g (NH₄)₂-citrate, 1.0 g Na-acetate, 5.0 g casamino acids (ex Difco), 6.7 g Yeast Nitrogen Base (ex Difco) and as a

carbon source 2 % glucose) incubated 24 hours at 30 °C was measured with the Haake Rotovisco RV100; system CV100 (sensor system ZA-30) at a shear rate of 300 s⁻¹. The viscosity of the 4 strains isolated from the Belgium salami turned 5 out to be approximately the same and ranged from 1.9 to 2.2 mPa.s. As a typical strain we have chosen the one showing the highest viscosity, produced when grown under the above mentioned conditions.

- 10 This strain was named *Lactobacillus sake* like strain O-1. In this specification the abbreviated forms "strain O-1" and "O-1" are also used.

15 Example 2 Physiological and taxonomical analysis of the exopolysaccharide producing *Lactobacillus sake* like strain O-1

The investigated strain O-1 is gram-positive, catalase-negative, immotile, and has rod-shaped bacteria occurring in short chains of in between 1 to 5 cells when grown in 20 MRS broth. This strain was able to ferment the following sugar substrates (determined with API 50 CHL): L-arabinose, ribose, galactose, D-glucose, D-fructose, D-mannose, N-acetyl glucosamine, esculin, salicin, cellobiose, lactose, and saccharose.

25 The taxonomical identification of the lactic acid bacteria isolated from the various fermented sausages as mentioned in Example 1 was carried out using polyacrylamide gel-electrophoresis of proteins. The strains were grown on MRS 30 agar in Roux flasks at 30 °C for 24 hours. Roux flasks were inoculated from a 24 hours grown MRS broth culture. Whole cell protein extracts were prepared as described previously (Kiredjan, 1986). Cells were lysed by sonication using a Labsonic 2000 sonicator (ex B. Braun, Melsungen, Germany) 35 with a needle probe tip (length 127 mm, diameter 4 mm) during 3 minutes in position "LOW" with 50 W output. Sodium dodecylsulphate polyacrylamide gel-electrophoresis was carried out using the procedure of Laemmli (1970), which

was modified as described by Kiredjan (1986). Registration of the protein electrophoretic patterns, normalization of the densitometric traces, grouping of strains by the Pearson product moment correlation coefficient (r) and
5 UPGMA (Unweighted Pair Group Method using Average linkage) cluster analysis were performed by the techniques described by Pot (1992), using the software package GELCOMPAR (version 2.0; L. Vauterin & P. Vauterin, commercially available at Applied Maths, Risquons-Toutstraat 38, B-8511
10 Kortrijk, Belgium).

This method was used for the identification of 32 strains that were isolated from the traditionally fermented Belgium salami as mentioned in Example 1. Of these 32 strains 4
15 were able to produce EPS, one of which was the above indicated strain O-1. For the identification of these 32 strains the protein patterns were compared with the protein patterns of 600 lactic acid bacteria reference strains which were stored in a database. In Figure 1 the UPGMA
20 dendrogram of the mean correlation coefficient (r), calculated between all pairs of strains, is presented. Both the investigated strains and the closely related reference strains are indicated, clearly showing that the strains from the Belgium salami occupied a taxonomic position
25 separate from the *Lactobacillus sake* reference strains. However, three reference strains which were phenotypically identified as *Lactobacillus sake* but showed aberrant protein patterns, were closely related to the strains isolated from the Belgian salami. These reference strains
30 were known as "Lactobacillus sake like" and therefore the strains isolated from the Belgian salami were also considered to be "Lactobacillus sake like".

**Example 3 Isolation and purification of the
exopolysaccharide produced by the *Lactobacillus*
sake like strain O-1**

5 A single colony of strain O-1 was selected from a MRS agar plate, transferred to fresh MRS broth and incubated overnight at 30 °C. Freshly prepared Semi Defined Medium (SDM-1), as described in Example 1, was inoculated with 2.5 % (v/v) of the overnight grown strain O-1 culture. This

10 inoculated SDM-1 was incubated for 24 hours at 30 °C without aeration. To remove proteins from the culture broth trichloroacetic acid (TCA) was added until a concentration of 4 % was reached. After gentle mixing the culture was allowed to stand for 30 minutes at room temperature. The

15 culture was centrifuged for 30 minutes at 27000 g and the clear supernatant was collected. The EPS produced was precipitated with 2.5 volumes of cold ethanol. The precipitate was collected, redissolved in approximately 5 % (v/v) of the original volume of water and dialysed against

20 demineralized water for 2 days at 4 °C. The water was refreshed twice a day. After dialysis a fractionated acetone precipitation was carried out. The bulk of the dissolved EPS was precipitated in the 50 % (v/v) acetone fraction. This fraction contained about 80 wt.-% of the

25 total amount of EPS and was 99 wt.-% pure, i.e. protein contamination < 1 wt.-%. When an ≈ 100 wt.-% pure EPS is required the material can be further purified by gel filtration on a column of Sephadryl S-500 (ex Pharmacia). The precipitate of this Example was redissolved in

30 demineralized water and lyophilized. The material was stored under dry conditions at 4 °C.

Example 4 Compositional analysis of the exopolysaccharide produced by the *Lactobacillus sake* like strain O-1

5 The sugar composition of the EPS produced by strain O-1 was determined. After methanolysis, followed by N-(re)acetylation and trimethylsilylation of the EPS the methyl glycosides of the different types of monosaccharides were determined by GC-MS. The EPS was composed of 60-70
10 wt.-% glucose and 30-40 wt.-% rhamnose.

Example 5 Rheological behaviour of both the exopolysaccharide produced by the *Lactobacillus sake* like strain O-1 and xanthan gum produced by *Xanthomonas campestris*.

15 The viscosity of a 1 % aqueous solution of the EPS produced by strain O-1 was determined as a function of the shear rate. All rheological measurements were carried out at 25 °C using a Haake Rotovisco RV100; system CV100; sensor system ZA-30. In Figure 2 the viscosity profile is compared with that of a 1 % aqueous solution of xanthan gum (ex Kelco). In Figure 2 it is clearly shown that the useful shear-thinning property of xanthan gum is also present in the O-1 EPS.

20 25 Detailed measurements at a shear rate of 3 s⁻¹, showed that the viscosity of a 1 % aqueous solution of the O-1 EPS was 2440 mPa.s, where the viscosity of a 1 % aqueous solution of xanthan gum was 1240 mPa.s. The viscosities measured at a shear rate of 300 s⁻¹ of a 1 % aqueous solution of O-1
30 EPS and of xanthan gum were determined as 70 and 54 mPa.s respectively, whereas the viscosities measured at a shear rate of 300 s⁻¹ of a 0.2 % aqueous solution of O-1 EPS and of xanthan gum were determined as 18 and 14 mPa.s, respectively.

Example 6 Optimization of the growth of the exopolysaccharide producing *Lactobacillus sake* like strain O-1

5 In order to study the physiology of the production of EPS by strain O-1 a defined growth medium was desired. Since MRS is a rich, non-specified medium (containing yeast extract, beef extract etc.) and the medium used in Example 1 resulted in a poor growth (optical density at 610 nm was
10 ≈ 1.2), a new Semi Defined Medium (SDM-2) was developed. For 1 litre SDM-2 the following components were weighed out: 10.0 g K_2HPO_4 , 12.4 g NaH_2PO_4 , 0.6 g $(NH_4)_2$ -citrate, 0.05 g $MnSO_4$, 5.0 g casamino acids (ex Difco), 10.0 g Bacto proteose pepton (ex Difco), 6.7 g Yeast Nitrogen Base (ex Difco) and 20 g glucose. The final pH of the medium was
15 6.5. After incubation of strain O-1 in this SDM-2 at 35 °C in non-shaken Erlenmeyer flasks without pH control the OD_{610} was 5.0, whereas growth in MRS resulted in a OD_{610} of 3.5-4.0. Anaerobic incubation in SDM-2 at 20 °C for 48
20 hours resulted in a OD_{610} of 6.5, whereas the viscosity of the culture broth was 3.8 mPa.s compared with a highest value of 2.2 when grown in SDM-1 (see Example 1). The concentration of the EPS in broth was 90 mg/l compared with a value of 30 mg/l when grown in SDM-1.

25

Example 7 *In situ* production of the exopolysaccharide of *Lactobacillus sake* like strain O-1 in a milk-based medium

300 g milk powder and 30 g Hysoy (ex Quest Bioproducts,
30 USA) were dissolved in water, up to an end volume of 3 l. It was pasteurised by incubating it for 1 minute at 90 °C. Subsequently, 0.3 mM $MnSO_4$ was added and after cooling to about 30 °C, the milk medium was inoculated with 1 % (v/v) of an overnight culture of *Lactobacillus sake* like strain
35 O-1, grown in the same medium. After incubating the culture for 18 hours at 30 °C, a highly viscous fermented milk was obtained, which was used in the production of a low-fat dressing, as shown in the following Example.

Example 8 Preparation of a dressing based on the in situ produced exopolysaccharide of *Lactobacillus sake* like strain O-1

A pre-emulsion phase was made by mixing equal volumes of
5 vinegar and a mixture of water, egg-yolk, edible oil and β -carotene at low shear. This pre-emulsion phase was mixed with an aqueous phase containing sorbate, sugar, salt, vinegar, citric acid and instant starch and subsequently the mixture was homogenised at high shear. The exact
10 composition of the mixtures depends on the properties, required for the final product. To this emulsion, the viscous fermented milk, the preparation of which was described in the preceding Example, was added at an end concentration of 10-20 % (v/v). The mixture was carefully
15 homogenised under low shear. A dressing having excellent body and taste was obtained.

Example 9

A spread can be obtained by preparing an aqueous phase
20 containing 2 wt% (on product) of the exopolysaccharide as described above and mixing 60 parts of the aqueous phase with 40 parts of a fat phase containing butterfat and 1% (on product) of monoglycerides.

Example 10, improved production of EPS

The following medium was used

5	Na ₂ HPO ₄	10	g/litre
	KH ₂ PO ₄	12	g/litre
	(NH ₄) ₂ -citrate	0.6	g/litre
	MnSO ₄	0.05	g/litre
	NZ-case Plus (exQuest)	20	g/litre
10	glucose	20	g/litre
	Yeast nitrogen base (ex Difio)	6.7	g/litre
	pH (NaOH)	5.8	
	water		balance

- 15 Lactobacillus sake 0-1 was anaerobically grown on this medium at 20°C while maintaining the pH at 5.8 for about 36 hours. Under these conditions 1350 mg EPS/l is produced. The structure of the EPS is determined by NMR and is as represented in figure 3 (ratio glucose: rhamnose 3:2, 0.425
20 0-acetyl groups per rhamnose unit, 0.5 1-phospho glycerol group rhamnose unit).

C L A I M S

1. Polysaccharide obtainable from a *Lactobacillus sake* like strain, preferably from *Lactobacillus sake* like strain O-1, which strain has been deposited as CBS 532.92.
2. Polysaccharide, comprising units of the monosaccharides rhamnose and glucose in a ratio of about 1:4 to about 1:1.
3. Polysaccharide according to claim 1 or 2, having a viscosity of at least 10 mPa.s when measured with a Haake Rotovisco RV100 viscosimeter in a concentration of 0.2 wt% in water at a shear rate of 300 s^{-1} , and/or of at least 1000 mPa.s when measured in a concentration of 1 % wt. in water at a shear rate of 3 s^{-1} or of at least 100 Pa.s when measured in a concentration of 1 wt% in water at a shear rate of 0.01 s^{-1} .
4. Polysaccharide according to claim 1, having shear-thinning properties in that the viscosity is reversibly reduced upon increasing the shear rates.
5. A process for producing a polysaccharide as claimed in any one of claims 1-4, comprising
 - (a) growing a *Lactobacillus sake* like strain capable of forming an exopolysaccharide (EPS) in a suitable medium under conditions whereby said EPS is formed, and optionally
 - (b) isolating the EPS formed.

6. A process for *in situ* producing a polysaccharide as claimed in any one of claims 1-4, comprising growing a *Lactobacillus sake* like strain in an edible medium under conditions whereby said EPS is formed until the number of said lactobacilli is in the order of 10^7 to 10^{11} per ml, preferably 10^9 to 10^{10} per ml.
7. *Lactobacillus sake* like strain capable of producing an EPS having at least one of the following properties
 - (1) comprising units of the monosaccharides rhamnose and glucose in a ratio of about 1:4 to about 1:1,
 - (2) giving a viscosity of at least 10 mPa.s when measured with a Haake Rotovisco RV100 viscosimeter in a concentration of 0.2 wt% water at a shear rate of 300 s^{-1} , or of at least 1000 mPa.s when measured in a concentration of 1 % wt. in water at a shear rate of 3 s^{-1} , or of at least 100 Pa.s when measured in a concentration of 1 wt% in water at a shear rate of 0.01 s^{-1} ; and
 - (3) having shear-thinning properties in that the viscosity is reversibly reduced upon applying higher shear rates.
8. *Lactobacillus sake* like strain O-1, deposited as CBS 532.92.
9. Food product comprising 0.01 - 15 wt% of a polysaccharide according to one or more of the claims 1-4.
10. Food product according to claim 9 selected from the group of spreads, dressings, creams, sauces, meat (products), cheese, puddings, ice-cream and bakery products.

UPGMA dendrogram of Pearson Product Moment Correlation coefficients (rx100) between SDS-PAGE protein patterns

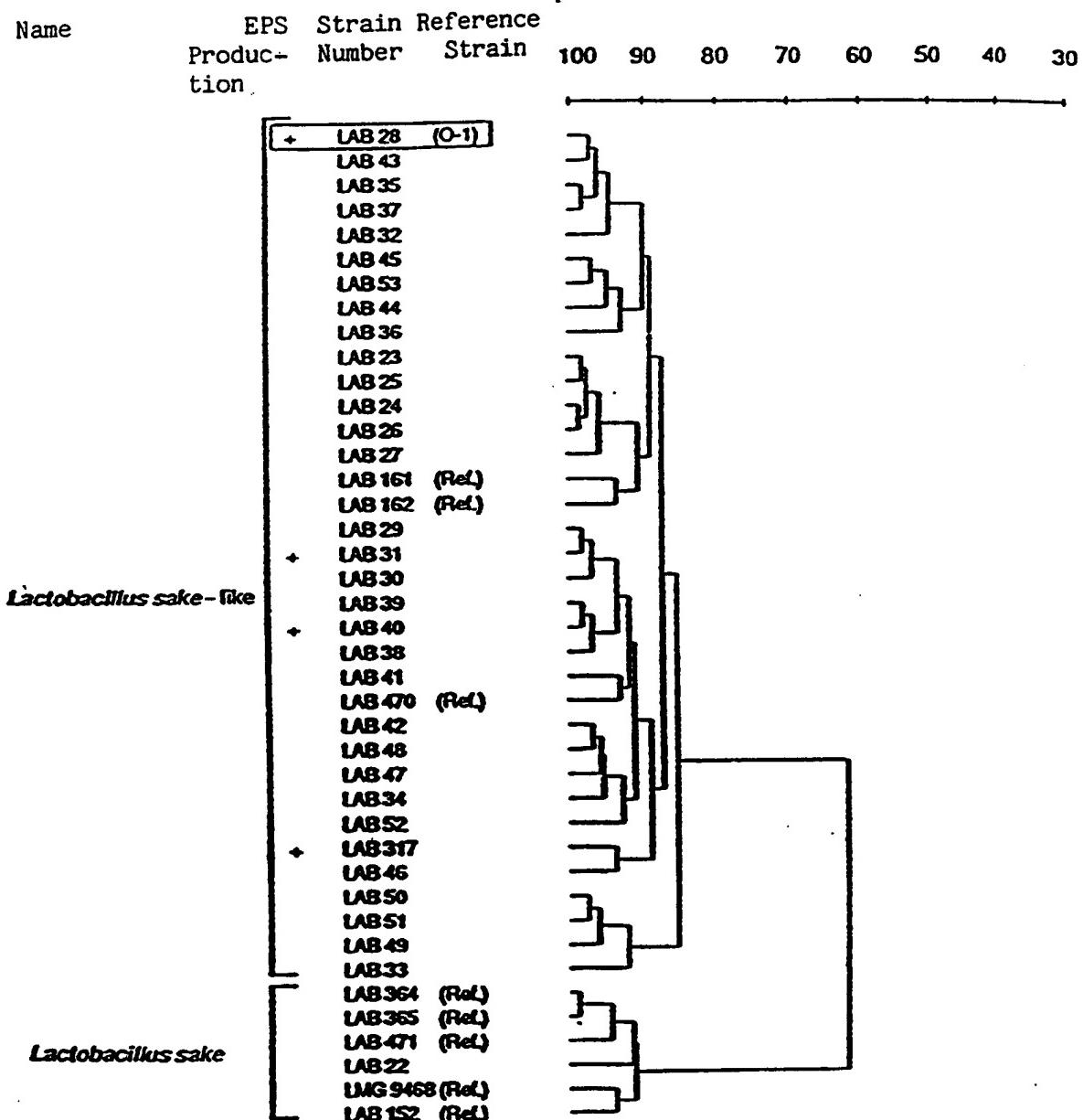
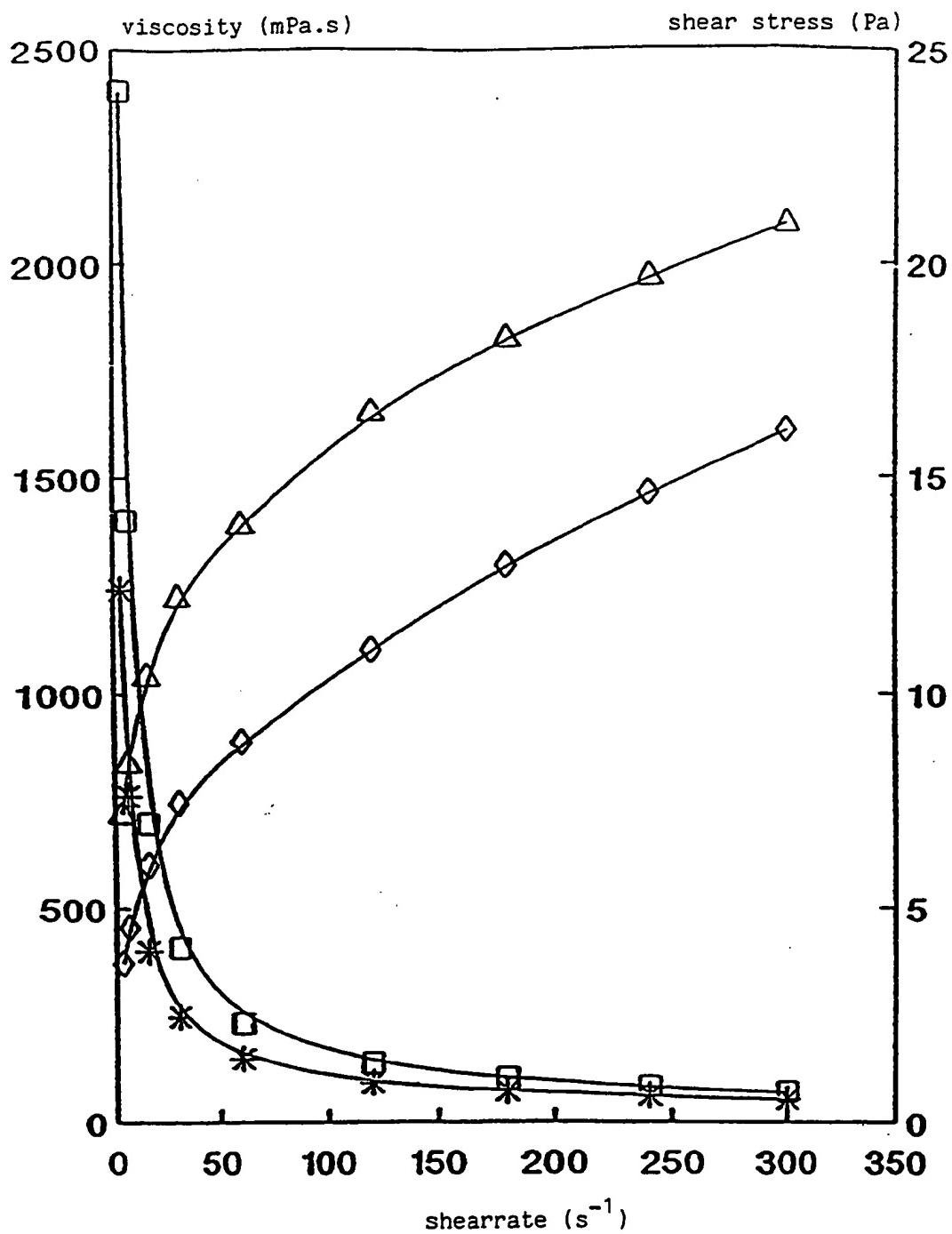


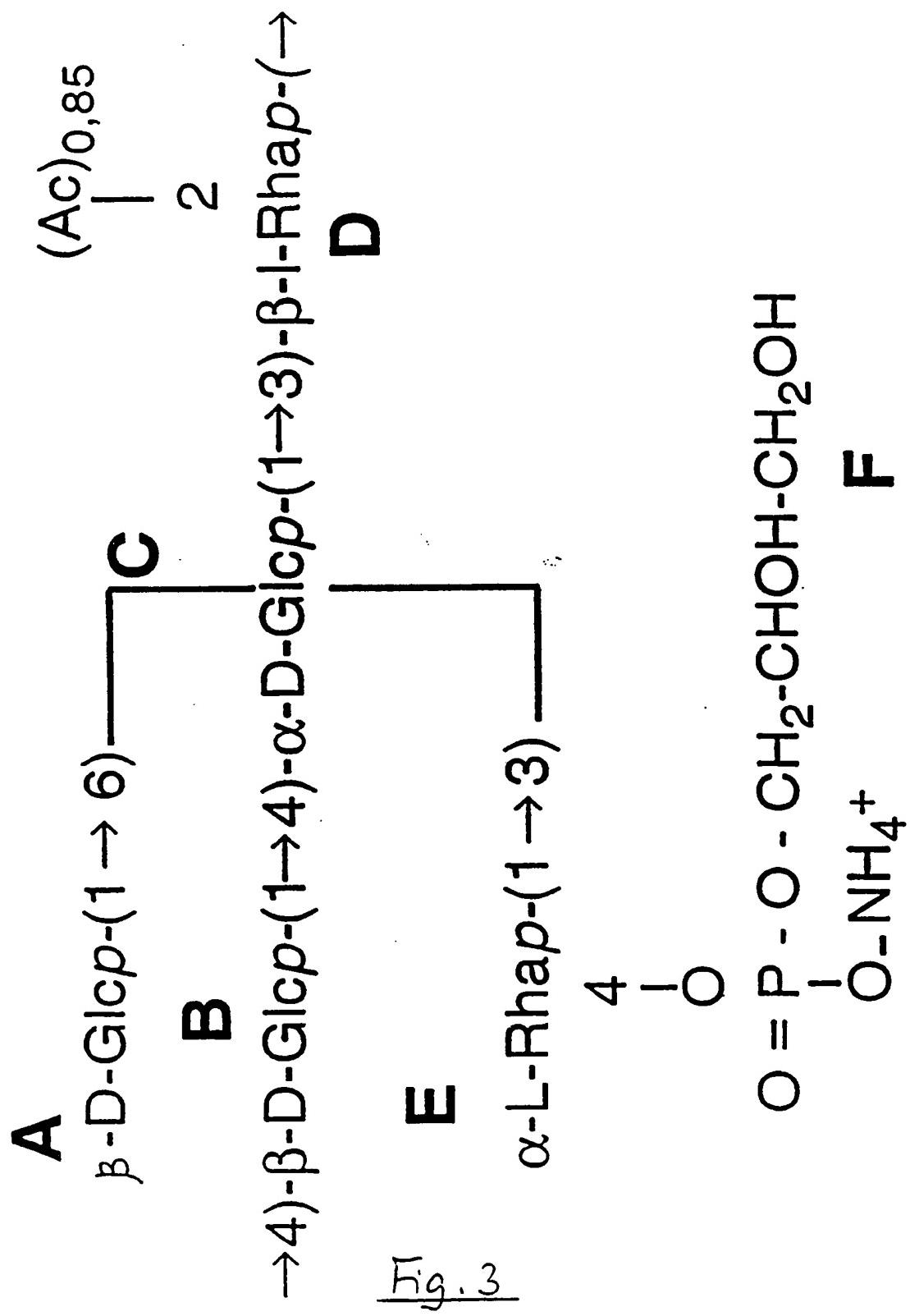
Fig. 1



—△— shear stress 1% O-1 EPS —□— viscosity 1% O-1 EPS
—◇— shear stress 1% xanthan —*— viscosity 1% xanthan

Fig. 2

3 / 3



INTERNATIONAL SEARCH REPORT

Internat'l Application No

PCT/EP 93/03338

A. CLASSIFICATION OF SUBJECT MATTER
 IPC 5 C12P19/04 C12N1/20 A23L1/24 // (C12N1/20, C12R1:225)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
 IPC 5 C12P C12R A23L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PATENT ABSTRACTS OF JAPAN vol. 12, no. 479 (C-552)(3326) 14 December 1988 & JP,A,63 196 521 (YAKULT HONSHA) 15 August 1988 see abstract ----- -/-	1

 Further documents are listed in the continuation of box C. Patent family members are listed in annex.

* Special categories of cited documents :

- *'A' document defining the general state of the art which is not considered to be of particular relevance
- *'B' earlier document but published on or after the international filing date
- *'L' document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *'O' document referring to an oral disclosure, use, exhibition or other means
- *'P' document published prior to the international filing date but later than the priority date claimed

- *'T' later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *'X' document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *'Y' document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- *'A' document member of the same patent family

Date of the actual completion of the international search

15 March 1994

Date of mailing of the international search report

25.04.94

Name and mailing address of the ISA
 European Patent Office, P.B. 5818 Patentstaan 2
 NL - 2280 HV Rijswijk
 Tel. +31 70 340 7000 Telex 31 651 eno nl

Authorized officer

INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 93/03338

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>CHEMICAL ABSTRACTS, vol. 115, no. 13, 30 September 1991, Columbus, Ohio, US; abstract no. 134184q, MIZOGUCHI, HARUHIKO ET AL. 'Separation of promotion factor on enzymic dissolution of rice from autolyzate of lactic acid bacteria.' page 805 ; see abstract & HAKKO KOGAKU KAISHI vol. 69, no. 4 , 1991 , JAPAN pages 211 - 217 ---</p>	1
A	<p>CHEMICAL ABSTRACTS, vol. 110, no. 5, 30 January 1989, Columbus, Ohio, US; abstract no. 36538k, HIGASHI, KAZUO ET AL. 'Nutritional requirement of hiochi bacteria under high ethanol concentration.VIII. Effect of ethanol on teichoic acid and polysaccharide in cell walls.' page 312 ; see abstract & NIPPON JOZO KYOKAISHI vol. 83, no. 10 , 1988 , JAPAN pages 697 - 702 ---</p>	1
A	<p>CHEMICAL ABSTRACTS, vol. 105, no. 23, 8 December 1986, Columbus, Ohio, US; abstract no. 205849s, CERNING, JUTTA ET AL. 'Isolation and characterization of exocellular polysaccharide produced by Lactobacillus bulgaricus.' page 322 ; see abstract & BIOTECHNOL. LETT. vol. 8, no. 9 , 1986 pages 625 - 628 -----</p>	1